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(54) Title: INTERACTION OF p53 WITH TRANSCRIPTION FACTOR DP-1

(57) Abstract

Complexes of p53 with the transcription factors DP-1 (and other members of the DP family) or E2F-1 (or other E2F proteins, up to and including E2F-5), are disclosed. These novel complexes can be used to assay for potential chemotherapeutic agents that are p53 agonists or antagonists, particularly agonists or antagonists which modulate progression through the cell cycle by disruption of the normal binding of p53 to DP-1 and/or E2F-1.

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INTERACTION OF p53 WITH TRANSCRIPTION FACTOR DP-1

This invention relates to complexes of the protein p53, which is a transcription modulator, with DP proteins (e.g. DP-1) or E2F proteins (e.g. E2F-1). In particular, the invention relates to use of these complexes in assays for potential therapeutic agents.

The cellular transcription factor DRTF1/E2F and the tumour suppressor protein p53 play important roles in controlling early cell cycle events. DRTF1/E2F is believed to co-ordinate and integrate the transcription of cell cycle regulating genes, for example those involved in DNA synthesis, with the activity of regulatory proteins, such as the retinoblastoma tumour suppressor gene product (pRb), which modulate its transcriptional activity. In contrast, p53 is thought to monitor the integrity of chromosomal DNA and when appropriate interfere with cell cycle progression, for example, in response to DNA damage.. Generic DRTF1/E2F DNA binding activity and transcriptional activation arise when a member of two distinct families of proteins interact as DP/E2F heterodimers, such as DP-1 and E2F-1. In many cell-types DP-1 is a widespread component of DRTF1/E2F DNA binding activity which when expressed at high levels oncogenically transforms embryonic fibroblasts.

The transition from G1 into S phase is an important regulatory point in cell cycle progression since that is when a number of genes need to be transcriptionally activated in order that cells may continue through the cell cycle. Many of these genes contain within their control sequences binding sites for the cellular transcription factor DRTF1/E2F which is widely believed to play an important role in regulating transcription during early cell cycle progression (La Thangue, 1994).

The potential role of DRTF1/E2F in cell cycle control is underscored by the properties of the proteins which are known to influence its transcriptional activity. For example, a group of proteins which negatively regulate the cell cycle, including the retinoblastoma tumour suppressor protein (pRb) and its relatives p107 and p130 (collectively known as pocket proteins) bind to and inactivate the transcriptional activity of DRTF1/E2F (Bandara and La Thangue, 1991; Chellappan *et al.*, 1991; Schwarz *et al.*, 1993; Cobrinik *et al.*, 1993). These interactions can be de-regulated in tumour cells, for example, through the action of viral oncoproteins, such as adenovirus E1a (Nevins, 1992), and furthermore are known to be temporally influenced by cell cycle progression (Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993). Another group of molecules, the cyclins and their catalytic regulatory subunits, which regulate cell cycle transitions, interact with DRTF1/E2F (Bandara *et al.*, 1991; Mudryj *et al.*, 1991; Devoto *et al.*, 1992; Lees *et al.*, 1992). Cyclins A, E and D together with an appropriate catalytic subunit are believed to influence the activity of pocket proteins (Hinds *et al.*, 1992; Sherr *et al.*, 1993), and direct phosphorylation by a cyclin A-cdk2 kinase reduces the DNA

binding activity of DRTF1/E2F (Dynlacht *et al.*, 1994; Krek *et al.*, 1994). Overall, the nature of the target genes together with the physiological properties of the afferent signalling proteins suggest that the activity of DRTF1/E2F plays a pivotal role in regulating and co-ordinating early cell cycle progression.

It is now known that generic DRTF1/E2F DNA binding activity defined in mammalian cell extracts results from an array of heterodimers made up from two distinct families of proteins, E2F and DP (E2F/DP heterodimers constituting physiological DRTF1/E2F). To date five members of the E2F family have been defined, from E2F-1 to E2F-5 which, in the context of an E2F/DP heterodimer, dictate the interacting pocket protein (Helin *et al.*, 1992; Ivey-Hoyle *et al.*, 1993; Kaelin *et al.*, 1992; Shan *et al.*, 1992; Lees *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Sardet *et al.*, 1995; Buck *et al.*, 1995; Hijmans *et al.*, 1995). The proteins E2F-4 and E2F-5 are the subject of International Patent Application Nos. PCT/GB95/00868 and PCT/GB95/00869, respectively. Three members of the DP family are known to exist namely DP-1, DP-2 and DP-3 (Girling *et al.*, 1993 and 1994; Ormondroyd *et al.*, 1995). From these, DP-1 is the most widespread member of DRTF1/E2F yet defined (Bandara *et al.*, 1993 and 1994) being for example, in all the various forms of DRTF1/E2F which occur during the cell cycle in 3T3 cells (Bandara *et al.*, 1994).

Both the E2F and DP proteins are endowed with growth-promoting activity since in a variety of assays they have been shown to possess proto-oncogenic activity (Singh *et al.*, 1994; Ginsberg *et al.*, 1994; Johnson *et al.*, 1994, Xu *et al.*, 1995; Jooss *et al.*, 1995). For example, over-expression of DP-1 or DP-2 together with activated Ha-*ras* causes transformation of rat embryo fibroblasts which, interestingly, is apparent in the absence of a co-transfected E2F family member (Jooss *et al.*, 1995).

Martin *et al.*, 1995, report that the proto-oncogene MDM2 is able to form complexes with the E2F family members E2F1 and DP-1. This interaction thus stimulates S-phase progression of the cell cycle. The MDM2 gene product is believed to downmodulate p53 function and the downmodulation of this tumour suprssor protein is believed to play a part in a number of tumour types where over-expression of MDM2 is found. Xiao et al., 1995 show an interaction between MDM2 and pRB.

In this application it is demonstrated that DP-1 exists in at least two distinct forms which differ in DNA binding properties. Further, we have found that p53 interacts with DP-1. Functionally, p53 regulates transcription driven by the DP-1/E2F-1 heterodimer by repression. Thus DP-1 is a common cellular target in two distinct pathways of growth control mediated through the activities of the pRb and p53 tumour suppressor proteins. Moreover, the integration of p53 and MDM2 with DP-1 defines a potential pathway through which p53 and MDM2 can influence cell cycle progression.

Thus the functional consequences of the interaction of p53 with a DP-1/E2F heterodimer is inactivation of transcription driven by the E2F binding site whereas, in contrast, the MDM2 protein activates DP-1/E2F-dependent transcription. Thus DP-1 appears to be a common cellular target in two distinct pathways of growth control, and these pathways, regulated by the pRb and p53 tumour suppressor proteins, now appear to be functionally integrated.

The invention is thus based on the surprising finding that p53 is able to interact with DP1, and thereby modulate the transactivation activity of the DP/E2F complexes.

Thus the invention in a first aspect provides a complex (preferably in a substantially isolated form) which comprises p53 and a DP or E2F protein.

The term "DP protein" is intended to encompass not only DP-1 but additionally DP-2 and DP-3 and related proteins of similar activity. By the term "E2F protein" it is intended to encompass the five known members of the E2F family, E2F-1 to E2F-5 inclusive, as well as related proteins of similar activity. However DP-1 protein of mammalian, preferably human or murine origin, is preferred.

These both additionally include mutants, allelic variants, fusions (with another protein) or species homologues of the naturally occurring proteins. They additionally include proteins that are at least 70% homologous to the naturally occurring protein, where that homologous protein is able to form a complex with p53.

Thus the expression "DP protein" comprises:

(a) a DP protein, such as DP-1, DP-2 or DP-3;

(b) a mutant, allelic variant or species homologue of (a);

(c) a protein at least 70% homologous to (a) or (b);

(d) a fragment of any of (a) to (c) capable of forming a complex with p 53 or MDM2;

(e) a fragment of any of (a) to (d) of at least 18 amino acids long; or

(f) a fusion protein comprising a protein or defined in any of (a) to (e) fused to another (e.g. heterologous) protein.

The expression "E2F protein" is to be construed likewise although of course in (a) above one would instead insert the E2F proteins, E2F-1 to E2F-5, inclusive. Mammalian, preferably human or murine, E2F-1 is preferred.

Mutants will possess one or more mutations which are additions, deletions, or substitutions of amino acids residues. Preferably these mutations will not affect, or not substantially affect, the structure and/or function and or properties of the protein. Mutants will generally still possess the ability to be able to complex with p53, as the context requires. Mutants can either be naturally occurring (that is to say, purified or isolated from a natural source) or synthetic (for example, by performing a site-directed mutagenesis on the encoding DNA). It will be apparent that the proteins used in the complexes of the invention can either be naturally occurring or recombinant.

The term "p53" likewise includes fragments, mutants, allelic variants and species homologues etc. in the same manner as described and defined for the DP and E2F proteins. Particular p53 mutants include those p53 mutants which are found in tumours, for example substitutions at R175, G245, R248, R249, R273, R282 and also mutants in the region 100-150.

For simplicity, p53 proteins (and their mutants, allelic variants and species homologues etc) will be referred to as transcriptional modulators. Proteins that fall within the terms "DP protein" and "E2F protein" are referred to as transcription factors. Thus, in complexes of the invention, the transcription modulator is (e.g. reversibly) bound to the transcription factor. Proteins unable to bind, or to complex with, one of the transcription modulator or transcription factor will not be included in complexes of the invention.

We have determined that DP-1 exists in two forms defined using immunochemical reagents. The two forms may be different by virtue of the degree of phosphorylation. p53 binds preferentially to the form and the resulting complex appears to contribute to the growth inactivating effects of DP-1.

It is possible to determine whether a transcription modulator or transcription factor will form a complex with the other by providing the two proteins and determining whether or not a complex has formed, for example by determining the molecular weight of the complex by methods such as SDS-PAGE or more preferably by using a tag or label to detect a fusion protein forming part of the complex.

A complex of the invention will be in substantially isolated form if it is in a form in which it is free of other polypeptides with which it may be associated in its natural environment (eg the body). It will be understood that the complex may be mixed with carriers or diluents which will not interfere with the intended purpose of the complex and yet still be regarded as substantially isolated.

The complex of the invention may also be in a substantially purified form, in which case it will generally comprise the complex in a preparation in which more than 90%, eg. 95%, 98% or 99% of the proteins in the preparation is a constituted by a complex of the invention.

An allelic variant will be a variant which will occur naturally in the same animal and which will function in a substantially similar manner to the proteins described herein.

Similarly, a species homologue will be the equivalent protein which occurs naturally in another species, and which performs the equivalent function in that species to the protein described herein. Within any one species, a homologue may exist as several allelic variants, and these will all be considered homologues.

Proteins at least 70% homologous to the naturally occurring protein are also contemplated for use in the complexes of the invention, as are proteins at least 80 or 90% and more preferably at least 95% homologous. This will generally be over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous amino acids. Methods of measuring protein homology are well known in the art and it will be understood by those of skill in the art that in the present context. Homology is usually calculated on the basis of amino acid identity (sometimes referred to as "hard homology").

Generally, mutants, fragments, allelic variants or species homologues thereof capable of forming a complex will be at least 10, preferably at least 15, for example at least 20, 25, 30, 40, 50 or 60 amino acids in length.

A complex of the invention may be labelled with a revealing or detectable label. The (revealing) label may be any suitable label which allows the complex to be detected. Suitable labels include radioisotopes, e.g. ^{125}I, enzymes, antibodies and linkers such as biotin. Labelled complexes of the invention may be used in diagnostic procedures such as immunoassays.

A complex (or labelled complex) according to the invention may also be fixed to a solid phase, for example the wall of an immunoassay dish.

A second aspect of the invention relates to a polynucleotide (suitably in a substantially isolated form) which comprises:

(a) a sequence encoding a transcription modulator and a transcription factor as defined in the first aspect;

(b) a sequence complementary to (a);

(c) a sequence at least 80% (e.g. 90%) homologous to a sequence in (a) or (b).

The polynucleotide may also comprise RNA. It may also be a polynucleotide which includes within it synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothionate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or lifespan of polynucleotides of the invention used in methods of therapy.

A polynucleotide of the invention will be in substantially isolated form if it is in a form in which it is free of other polynucleotides with which it may be associated in its natural environment (usually the body). It will be understood that the polynucleotide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polynucleotide and it may still be regarded as substantially isolated.

A polynucleotide according to the invention may be labelled, e.g. with a revealing or detectable label, by conventional means using radioactive or non-radioactive labels, or may be cloned into a vector.

Polynucleotides, such as a DNA polynucleotides according to the invention, may be produced recombinantly, synthetically, or by any means available to those of skill in the art. It may be also cloned by reference to the techniques disclosed herein.

The invention includes a double stranded polynucleotide comprising a polynucleotide according to the invention and its complement.

A third aspect of the invention relates to an (eg. expression) vector suitable for the replication and expression of the polynucleotide, in particular a DNA or RNA polynucleotide, according to the second aspect of the invention. The vector may be, for example, a plasmid, virus or phage vector provided with an origin of replication, optionally a promoter for the expression of the polynucleotide and optionally a regulator of the promoter. The vector may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. The vector may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used *in vivo*, for example in a method of gene therapy.

Vectors of the third aspect are preferably recombinant replicable vectors. The vector may thus be used to replicate the DNA. Preferably, the DNA in the vector is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by a host cell. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences. Such vectors may be transformed or transfected into a suitable host cell to provide for expression of component proteins in complexes of the invention.

The vector of the invention may comprise separate promoters operably linked to the sequences encoding the transcription modulator and the transcription factor or the vector may comprise a single coding sequence comprising both the modulator and factor. In this event, the factor and modulator sequences may be in either order. The sequences are optionally linked by a further sequence such as a linker sequence designed to allow the two encoded polypeptide sequences to fold in a manner found in nature or it may be a linker sequence which can be selectively cleaved allowing the two encoded polypeptide sequences to be separated. An example of the former type of linker includes an IgG hinge region whereas cleavable sequences of the latter type are well known per se in the art.

A fourth aspect of the invention thus relates to host cells transformed or transfected with the vectors of the third aspect, or able to express DNA sequence(s) encoding both the transcription modulator and transcription factor, as defined for the first aspect. This may allow for the replication and expression of a polynucleotide according to the invention. The cells will be chosen to be compatible with the vector and may for example be bacterial, yeast, insect or mammalian. Preferred hosts include 3T3 and SAOS-2 cells (the latter cells lack functional retinoblastoma (pRb) protein and p53).

The host cells of the fourth aspect express, and preferably secrete, both a transcription modulator and transcription factor, those terms already having been defined in the first aspect. Preferably the transcription modulator and factor will combine to form a complex of the first aspect, such interaction occurring either inside the cell or outside in the medium surrounding the host cells (if any).

Such expression can be achieved either by using a single vector, such as of the third aspect, which encodes both a transcription modulator and a transcription factor. Alternatively, a host cell can be transformed or transfected with two or more vectors. A first vector will encode the transcription modulator, while a second vector will encode the transcription factor. Both vectors will then be co-transfected into a host cell to produce the cells of the fourth aspect.

A polynucleotide according to the invention may also be inserted into the vectors described above in an antisense orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense polynucleotides may also be produced by synthetic means. Such antisense polynucleotides may be used in a method of controlling the levels of the transcription modulator, a DP protein and/or E2F protein in a cell. Such a method may include introducing into the cell the antisense polynucleotide in an amount effective to inhibit or reduce the level of translation of the E2F mRNA into protein. The cell may be a cell which is proliferating in an uncontrolled manner such as a tumour cell.

Thus, in a fifth aspect the invention provides a process for preparing a complex according to the invention which comprises cultivating a host cell of the fourth aspect, preferably transformed or transfected with an (expression) vector of the third aspect, under conditions providing for expression of coding sequence(s) encoding the component proteins of the complex, allowing the transcription modulator to bind to the transcription factor, and recovering the complex.

A sixth aspect of the invention relates to complexes of the first aspect, a polynucleotide of the second aspect, vectors of the third aspect and host cells of the fourth aspect for use in medicine. In other words, all such substances (from the first to fourth aspects inclusive) find use in a method for treatment of the human or animal body by therapy.

The uses contemplated are the inhibition of cell growth, or the interference with transcription. End uses here would therefore include the treatment of proliferative diseases, such as cancer.

The transcription modulator need not be the naturally occurring p53 protein, as has been explained for the first aspect. Indeed, using the screening methods of the invention (which are discussed later) substances other than the naturally occurring proteins, which have similar properties, can be identified. These too may then be used in the same or similar manner to the p53 protein themselves.

Thus, according to a seventh aspect of the present invention there is provided a pharmaceutical or veterinary composition comprising a complex of the first aspect, a polynucleotide of the second aspect, a vector of the third aspect, or a host cell of the fourth aspect, together with a pharmaceutically, or veterinarily, acceptable carrier or diluent, respectively.

Pharmaceutically or veterinarily acceptable carriers or diluents include those used in formulations suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural)

administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

For example, formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostatis and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the polypeptide to blood components or one or more organs.

Complexes according to the invention or transcriptional modulators may be used for the treatment, regulation or diagnosis of conditions, including proliferative diseases, in a mammal including man. Such conditions include those associated with abnormal (eg at an unusually high or low level) and/or aberrant (eg due to a mutation in the gene sequence) expression of one or more transcription factors such as the DP or E2F proteins or related family members. Treatment or regulation of conditions with the above-mentioned complexes or peptides will usually involve administering to a recipient in need of such treatment an effective amount of that complex or peptide as appropriate.

Vectors carrying a polynucleotide according to the invention or a nucleic acid encoding a complex according to the invention may be used in a method of gene therapy. Such gene therapy may aim to repress or inhibit cell growth and so treat uncontrolled proliferation of cells, for example a tumour cell. Alternatively the complexes may be used to encourage or activate cell growth. Methods of gene therapy may also include delivering to a cell in a patient in need of treatment an effective amount of a vector capable of expressing in the cell either an antisense polynucleotide of the invention in order to inhibit or reduce the translation of E2F or DP mRNA into the corresponding protein which may interfere with the binding of an E2F protein to a DP protein or a related family member.

Such a vector is suitably a viral vector. The viral vector may be any suitable vector available in the art for targeting tumour cells. For example, Huber et al (Proc. Natl. Acac. Sci. USA (1991) 88, 8039) report the use of amphotrophic retroviruses for the transformation of hepatoma, breast, colon or skin cells. Culver et al (Science (1992) 256; 1550-1552) also describe the use of retroviral vectors in virus-directed enzyme prodrug therapy, as do Ram et al (Cancer Research (1993) 53; 83-88).

Englehardt et al (Nature Genetics (1993) 4; 27-34 describe the use of adenovirus based vectors in the delivery of the cystic fibrosis transmembrane conductance product (CFTR) into cells.

According to an eighth aspect of the invention there is provided:

(a) a complex of the first aspect;

(b) a polynucleotide of the second aspect;

(c) a vector of the third aspect; or

(d) a host cell of the fourth aspect of the invention;

in the manufacture of a medicament for treating uncontrolled proliferation of cells, for example in the treatment of cancer, viral disease, heart disease, self proliferation disorders as well as auto-immune disorders such as psoriasis.

In another aspect, the invention provides a novel assay for identifying putative chemotherapeutic agents for the treatment of proliferative or viral disease which comprises bringing into contact a DP protein, an E2F protein and a putative chemotherapeutic agent, and measuring the degree of inhibition of formation of the DP/E2F protein complex caused by the agent. It may not be necessary to use the complete DP and/or E2F protein in the assay, as long as sufficient of each protein is provided such that under the conditions of the assay in the absence of agent, they form a heterodimer.

The cloning and sequencing of DP-1 (and E2F 1,2 and 3) are known in the art and methods for the recombinant expression of these proteins will be known to those in the art.

In a ninth aspect of the invention there is provided a screening method or assay for identifying potential or putative chemotherapeutic agents, the method or assay comprising providing a transcription modulator and a potential or putative chemotherapeutic agent, and measuring the extent to which the agent is able to bind to the transcription modulator, or whether the agent forms a complex, of the invention with the modulator.

The ninth aspect also extends to a similar method where the transcription modulator is replaced by a transcription factor. The transcription modulators and factors are as defined for the first aspect of the invention.

Thus, in the tenth aspect the invention provides a screening method or assay for identifying potential or putative chemotherapeutic agents, the method or assay comprising:-

(A) providing the following components:

(i) a DP protein and/or an E2F protein;

(ii) a p53 polypeptide; and

(iii) a potential or putative chemotherapeutic agent;

and bringing them into contact under conditions in which the components (i) and (ii) in the absence of (iii) form a complex; and

(B) measuring the extent to which component (iii) is able to disrupt or interfere with the complex, inhibit or encourage the binding of components (i) or (ii), or affect the activity of the complex.

In the assays, any one or more of the components may be labelled, eg with a radioactive or colorimetric label, to allow measurement of the result of the assay. Potential chemotherapeutic agents include transcription modulators and factors as described in the first aspect of the invention.

Mutants, homologues and fragments of the DP and E2F proteins have been defined earlier in a corresponding manner to the mutants, homologues and fragments of the MDM2 and p53 transcription modulator proteins, all of which find use in the assays of the invention.

Such an assay can be performed without the need for a DP protein. However, if both a DP and E2F protein are present the complex of (i) and (ii) may be measured, for example, by its ability to bind an E2F DNA binding site *in vitro*. Alternatively, the assay may be an *in vivo* assay in which the ability of the complex to activate a promoter comprising an E2F binding site linked to a reporter gene is measured. The *in vivo* assay may be performed in yeast, insect, amphibian or mammalian cells.

It will thus be seen that the assays of the invention extend to both to two component assays (in the ninth aspect) as well as three component assays (as in the tenth aspect).

In either a two component or three component system, but preferably the former, one or both of the components may comprise a fusion protein. That fusion protein, as mentioned earlier, is encompassed by the terms p53, DP and E2F proteins.

For example, a first protein of interest may be fused to a protein comprising a foreign DNA binding domain, such as a GAL-4 domain. That protein on its own would be inactive, and although it would bind DNA, it would not activate transcription. A second protein of interest (for example a DP protein, if the first protein was a transcription modulator) may then be fused to be a protein comprising a foreign activation domain, such as a GAL-4 activator. In the absence of the chemotherapeutic agent, the first and second proteins of interest (which would usually be a transcription modulator and a transcription factor) will form a complex. When respective fusion proteins are present, such as a foreign DNA binding domain and foreign activation domain, they will

cooperate to cause activation. The affect of the chemotherapeutic agent on this process can then be monitored. The agent will usually be added to medium surrounding cells, while inside the cell the two fusion proteins are expressed.

Thus the proteins that find use in the complex of the invention may themselves be fusion proteins. They be fused with another (different) protein which can act as a label or tag for detection of that fusion protein. Suitable tags include GST and a series of histidines residues (e.g. six), the latter being able to bind nickel.

Such fusion proteins may be used to purify the protein, or allow it to be used in assays. The fusion protein or tag may have an affinity for another protein or substance (which may be present on a column) or an antibody. For example, if the protein is fused to GST, this may aid its purification using glutathione sepharose ™ beads. Alternatively an antibody specific for the tag on the fusion protein may be employed.

Candidate therapeutic agents which may be measured by the assay include not only the transcription modulators and transcription factors mentioned in the first aspect, but in particular fragments of 10 or more amino acids of:

(a) a p53 protein;

(b) an allelic variant or species homologue thereof; or

(c) a protein at least 70% homologous to (a).

The assays of the invention can be performed using standard techniques, the components (i) and (ii) being taken from known, publicly available sources. One preferred method, however, in values providing the proteins in recombinant form, expressed by a host cell (e.g. of the fourth aspect). Host cells can be transfected or transformed in the assay performed *in vitro*.

The invention contemplates a number of assays. Broadly, these can be classified as follows.

1. Performing an assay to identify a substance that affects the interaction between the transcription modulator and the transcription factor. Such substances may interfere with or alter the transactivation repressing properties of p53. Such substances may be more effective than the natural molecules for themselves. For example, they may be able to repress transcriptional activity more effectively than p53. On the other hand, they may have lower levels of transcription repressing activity than p53. Such substances may therefore act as either agonists or antagonists of p53, as appropriate. The substances may interfere with the interaction or binding between the transcription

modulator and transcription factor, or alternatively competitively inhibit this interaction, or the formation of a complex between the transcription modulator and factor, by mimicking either of the transcriptional modulator or factor proteins.

Such an assay may be particularly useful in identifying compounds which are agonists of p53 or other substances which promote the presence in the cell of the form of DP-1 recognised by p53 which is likely to be growth inhibiting. For example such compounds may bind to DP-1 in a manner which mimics p53. Alternatively the compound may promote the binding of p53 to DP-1. Such assays may also be used with p53 mutants such as those described above to assay for compounds which restore the ability of such p53 mutants to bind to DP-1 where such p53 mutants have lost the ability to do so in tumour cells.

2. Conducting an assay to find an inhibitor of an E2F protein *trans*-activation (that is to say, inhibition of activation of transcription). This inhibitor may therefore inhibit binding of an E2F protein to DNA (usually at the E2F binding site). Potentially suitable inhibitors are proteins, and may have a similar or same effect as p53. Thus suitable inhibitory molecules may comprise fragments, mutants, allelic variants, or species homologues of p53 in the same manner as defined for complexes of the first aspect.

3. Assaying for inhibitors of (hetero)dimerisation which mimic the binding of p53 to DP-1. The binding of DP-1 to an E2F protein promotes cell proliferation and thus p53 competes with E2F for binding to DP-1. Such inhibitors may prevent dimerisation of an E2F protein (eg. E2F-1) with a DP protein, such as DP-1. The assay can measure the degree of inhibition of binding by determining the ability of p53 and/or E2F to compete with each other and the potential inhibitor. Of course the inhibitor can be a fragment, mutant, allelic variant or species homologue of a DP or E2F protein as defined for the complexes of the first aspect.

The invention thus contemplates the identification of substances for the treatment or prophylaxis of diseases that are based on the uncontrolled proliferation of cells, or where uncontrolled proliferation is an important or essential pathological aspect of the disease. This includes cancer, viral disease, self proliferation itself as well as auto immune diseases such psoriasis. Compounds contemplated here would include p53 agonists.

One may also wish to temporarily inhibit the growth of dividing cells, for example haematopoietic stem cells and/or bone marrow cells. In these aspects one is generally seeking to prevent, inhibit or interfere with the activity of an E2F protein.

In contrast some diseases and conditions can be treated by increasing E2F expression, for example by promoting or inducing overexpression. That could be achieved through the use of p53 antagonists. This preferably results in apoptosis, sometimes known as programmed cell death. Overexpression of the E2F protein can result in death of the cell (Qin *et al* ,1994) and therefore this aspect can also be used in the treatment of cancer. Thus E2F and p53 appear to modulate the activity of each other and disruption of the interaction by antagonists may promote the observed apoptotic effects of E2F.

The invention will now be described by way of example with respect to the accompanying Examples.

EXAMPLE 1

Immunochemistry:

Monoclonal antibodies 421 and SMP14 have been previously described (Harlow *et al.*, 1981; Picksley *et al.*, 1994). Anti-DP-1(A) is a rabbit polyclonal anti-peptide serum raised against a peptide representing an N-terminal region in DP-1, and has been previously described (Girling *et al.*, 1993; Bandara *et al.*, 1994). Anti-DP-1(D) rabbit polyclonal anti-peptide serum (Bandara *et al.*, 1994) and monoclonal antibody 32.3 were raised against a peptide representing a C-terminal region (residues 385 to 400) in DP-1. Immunoblotting with anti-DP-1(A), anti-DP-1 (D) or 32.3 was performed as previously described. Either the homologous peptide, or a control unrelated peptide, peptide 1, was added to assess specificity.

For immunoprecipitation, cells were harvested in LSL buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.1% NP40, 2μg/ml aprotinin, 0.5mM PMSF), to which 32.3 was added in the presence of peptide and incubated for 1hr on ice. Immune complexes were collected with protein A-Sepharose and washed extensively (at least 3 times) in LSL buffer, released in SDS sample buffer, electrophoresed and immunoblotted with anti-DP-1 (A). The procedure for sequential immunoprecipitation and immunoblotting has been previously described (Bandara *et al.*, 1993).

For the anti-DP-1 immuno-affinity chromatography, 2ml anti-DP-1(A) Ab was precipitated with ammonium sulphate (45%) and exhaustively dialysed in 10mM sodium phosphate (pH7.5). The resulting immunoglobulin was coupled to 3ml CNBr-activated Sepharose (as recommended by the manufacturer) and incubated with 3ml F9 EC extract for 24h at 4°C. The column was washed with NEP buffer (Girling *et al.*, 1993) containing 0.5% NP40, pre-elution buffer and bound proteins eluted using 0.1M glycine (pH2.5). Samples were collected and neutralized. The peak fractions were precipitated with trichloroacetic acid and washed with acetone before being solubilized in SDS sample buffer and immunoblotted with either 32.3 or SMP14.

Gel retardation:

Gel retardation with F9 EC cell extracts using an E2F binding site taken from the adenovirus E2a promoter was performed as described previously (Girling *et al.*, 1993). Monoclonal antibody 32.3 was added to the binding reaction, together with competing peptide, and incubated for 10 min at 30°C. To assess the DRTF1/E2F DNA binding activity immunoprecipitated by 32.3, immunoprecipitations were performed as described above in the presence of either the homologous or unrelated peptide. Competing homologous peptide in LSL was added after washing the immunoprecipitate in LSL buffer, the supernatant being subsequently assayed for DRTF1/E2F DNA binding activity.

The effect of p53 on the E2F-site binding activity of the E2F-1/DP-1 heterodimer was assayed using *in vitro* transcribed and translated DP-1 (pG4DP-1; 4) and E2F-1 (pSP72;27). *In vitro* transcription and translation was carried out in a TNT T7/SP6 coupled reticulocyte lysate system (promega). pH6-mmp53 wt encodes a His-tagged complete mouse p53 protein (kindly supplied by Gunnar Weidt and Wolfgang Deppert). His-tagged murine p53 was purified from a 500ml pellet of IPTG-induced bacterial culture. The bacterial pellet was resuspended in 10mls denaturing buffer (100mM sodium phosphate, 10mM tris base, 6.0M guanidine hydrochloride, 30mM imidazole) pH 8.0, and gently stirred for two hours at room temperature. $MgCl_2$ was added to a final concentration of 5mM and cellular debris were cleared by repeated centrifugation at 4°C. 400μl of nickel agarose (solid) QIAGEN) was added to the supernatant and rotated for one hour at room temperature. The resin was washed stepwise with two 50ml volumes each of; denaturing buffer pH8.0, denaturing buffer pH 6.4 and renaturing buffer (25mM sodium phosphate pH 7.0, 300mM NaCl, 10mM β-mercaptoethanol) containing 1M then 0.1M and lastly no guanidine hydrochloride. Protein was eluted off the resin by sequential washes with imidazole buffer (150 mM imidazole, 100mM NaCl, 50mM Tris-HCl pH 8.0) and analysed by SDS-PAGE. An equal amount of heat-denatured or non-denatured His-tagged p53 was added to the binding reaction.

Fractionation:

Heparin-Sepharose and E2F binding site-affinity chromatography of F9 EC cell extracts was performed as previously described (Girling *et al.*, 1993). Fractions were assayed for DRTF1/E2F DNA binding activity and immunoblotted as described.

Binding assay for p53:

pH6-mmp53 wt encodes a His-tagged complete mouse p53 protein (kindly supplied by Gunnar Weidt and Wolfgang Deppert). pH6-mmp53, GST-pRb (Bandara *et al.*, 1991) and GST alone were induced and purified by conventional procedures. For the *in vitro* binding assay, 15μl of fusion protein bound to the appropriate agarose (glutathione-agarose or nickel-agarose) was incubated with F9 EC whole

cell extract with constant rotation for 2hrs at 4°C. The suspension was centrifuged and repeatedly washed with LSL buffer, resuspended in SDS loading buffer and immunoblotted with anti-DP-1(A).

Binding assay for DP-1:

GST-DP-1 encodes a complete DP-1 protein fused to GST in pGEX-3X and was induced and purified by conventional procedures. E2F-1, E2F-4 and wild-type p53 were transcribed and translated in the presence ^{35}S methionine in a TNT coupled lysate (Promega) as recommended by the manufacturer. For the *in vitro* binding assay in Fig. 5, fusion protein was added to the translate in PBSA containing 1mM EDTA, 1mM DTT, 0.5% Tween 20 and incubated for 30 min at 30°C. Glutathione beads were subsequently added and incubated for a further 30 min. Beads were collected and washed four times in the same buffer before being solubilized in SDS ample buffer. In order to map the region in p53 to which DP-1 binds a panel of p53 mutants were made. GST-p53 1-73, 1-143 and 1-235 were made by PCR using human p53 as template 9php53Cl; 50). PCR products were cloned in frame into a pGEX vector (Pharmacia). Fusion proteins were induced and purified by conventional procedures. For *in vitro* binding reactions approximately 10μg of GST or GST-p53 fusion protein bound to glutathion-agarose beads was added to 15μl of *in vitro* translated DP-1 in lysis buffer (50mM Tris pH 8.0, 150mM NaCl, containing 10mg/ml lysozyme, 0.5mM PMSF, 50μg/ml leupeptin, 50μg/ml protease inhibitor, 50μg/ml aprotinin and 40mM DTT). After incubation for 2.5h at 4°C the beads were collected and washed 4 times in lysis buffer. Proteins were released in SDS sample buffer, electrophoresed and immunoblotted with anti-DP-1(A) or anti-DP-1 (D).

Binding assay for MDM2:

pGST-MDM2 encodes a complete MDM2 protein fused to GST in pGEX-3X and was induced and purified by conventional procedures. DP-1 and wild-type p53 were transcribed and translated in the presence ^{35}S methionine in a TNT coupled lysate (Promega) as recommended by the manufacturer. For the *in vitro* binding assay, fusion protein was added to the translate in PBSA containing 1mM EDTA, 1mM DTT, 0.5% Tween 20 and incubated for 30 min at 30°C. Glutathione beads were subsequently added and incubated for a further 30 min. Beads were collected and washed four times in the same buffer before being solubilized in SDS sample buffer.

Transient transfection:

The reporter construct p3xWT-GL, p3xMT-GL, pCMV-βgal, pCMV-E2F-1 and pCMV-DP-1 have all been described previously (Girling *et al.*, 1994; Jooss *et al.*, 1995). pC53-SN3 encodes wild-type p53 driven by the CMV enhancer/promoter region (Baker *et al.*, 1990). pJ4Ω-MDM2 encodes the complete MDM2 protein (Oliner *et al.*, 1992). The total amount of DNA in each transfection was made up with empty vector which, in the case of the p53 titration was with pCMV-neoBam, and for

the MDM2 titration waswith pJ4Ω. Cells were transfected by the conventional calcium phosphate procedure. Luciferase and β-galactosidase assays were peformed as described previously (Girling *et al.*, 1994). Each treatment was performed in duplicate.

Results

Distinct forms of DP-1.

Two distinct DP-1 polypeptides of 55kD can be resolved during cell cycle progression in 3T3 cells, referred to as p55L (lower) and p55U (upper), p55L appearing towards the end of G1 as cells begin to enter S phase (Bandara *et al.*, 1994). In order to characterise the two forms of p55 in greater detail an anti-peptide was prepared monoclonal antibody, 32.3, which recognises p55L. By immuno-blotting extracts prepared from a synchronous cultures of F9 EC cells a polyclonal antiserum, anti-DP-1 (A), revealed both forms of p55 in contrast to monoclonal antibody 32.3 which defined p55L. Further evidence that 32.3 recognises p55L was provided by immunoprecipitating with 32.3 in the presence and absence of the homologous peptide, and subsequently probing the immunoprecipitate with polyclonal anti-DP-1(A). The immunoprecipitated polypeptide co-migrated with p55L.

The effect of 32.3 on DRTF1/E2F DNA binding activity was examined. In extracts prepared from F9 embryonal carcinoma (EC) cells (obtained from the European Culture Collection) the addition of 32.3 to the binding reaction caused an almost complete shift of DRTF1/E2F compared to the reaction performed in the presence of a homologous peptide; similar results were observed in extracts prepared from a wide variety of other types of cells. Furthermore, 32.3 immunoprecipitated DRTF1/E2F DNA binding activity from F9 EC cell extracts in which p55L was the predominant form of DP-1 present in the immunoprecipitate. These data suggest that p55L is a major component of DRTF1/E2F DNA binding activity.

In order to substantiate this idea the chromatographic properties of p55U and p55L were studied during the fractionation of F9 EC cell extracts, and the presence of each polypeptide with the presence of DRTF1/E2F DNA binding activity within each fraction was correlated. Extracts prepared from F9 EC cells were fractionated over heparin-Sepharose and subsequently assayed for DNA binding activity by gel retardation. Both p55U and p55L were present in the F9 EC cell extract, although an analysis performed on fractions after passage over heparin-Sepharose indicated that p55L, rather than p55U, correlated with DRTF1/E2F DNA binding activity. Thus, p55U was predominant in fractions which lacked DNA binding activity. In contrast, DRTF1/E2F DNA binding activity both before and after further purification by E2F binding site-affinity chromatography (Girling *et al.*, 1993), correlated with p55L. More rounds of E2F binding site-affinity chromatography did not alter the correlation.

When fractions containing p55L and p55U (with and without DRTF1/E2F DNA binding activity) were added together the original composition of p55U and L in the unfractionated cell extract was reconstituted. These data, combined with the results derived from the studies performed with monoclonal antibody 32.3, suggest that p55L is present in DRTF1/E2F DNA binding activity and, further, that p55U is likely to be a form of DP-1 which either cannot bind or binds a less efficiently to the E2F site.

DP-1 associates with p53:

A number of polypeptides with distinct molecular weights were detected when different polyclonal anti-DP-1 peptide antisera were used to sequentially immuno-precipitate DP-1 from ^{35}S-methionine radiolabelled extracts prepared from F9 EC cells. Thus, anti-DP-1(A) specifically immunoprecipitated a group of polypeptides with a range of molecular weights. When the anti-DP-1(A) immunoprecipitate was released and subsequently re-immunoprecipitated with anti-DP-1(D), which possesses similar specificity to 32.3 for p55L, a subset of the anti-DP-1(A) associated polypeptides were apparent. These data suggest therefore that a number of cellular polypeptides associate with p55U.

In order to characterise these DP-1-associated polypeptides in greater detail it was assessed whether antisera directed against previously identified polypeptides with similar molecular weights recognised them. A range of antisera were studied, two of which were found to be of particular interest. Thus, when an anti-p53 monoclonal antibody, 421, was used in an immunoprecipitation and subsequently immunoblotted with anti-DP-1(A), p55 was revealed. However, p55U rather than p55L preferentially co-immunoprecipitated with p53. To substantiate this result, further evidence for an interaction between p53 and DP-1 was obtained from an *in vitro* binding assay in which a p53 fusion protein was incubated in an F9 EC cell extract. In these conditions, p53 specifically associated with DP-1 in the F9 extract. As expected, an pRb fusion protein known to bind to DRTF1/E2F in F9 EC cell extracts (Bandara *et al.*, 1991) interacted with DP-1 in the same assay conditions. Overall, these results suggest that p53 associates with DP-1 in physiological conditions and, further, the preferred form of DP-1 that interacts with p53 is p55U.

Similar experiments were performed with a monoclonal antibody recognising MDM2, encoded by the *MDM2* oncogene, which is widely believed to be a transcriptional antagonist of p53 *trans* activation (Oliner *et al.*, 1992). These experiments (data not shown) confirmed the interaction which has also been reported by Martin *et al.*, 1995, and Xiao et al., 1995.

The efficiency of interaction of DP-1 and MDM2 was compared with the binding of p53 and MDM2. An assay was used in which the ability of MDM2, expressed as a GST fusion protein, to bind to *in*

vitro translated DP-1 and p53 was assessed. It was found that p53 and MDM2 were able to associate in the conditions of this assay and likewise, DP-1 could interact with MDM2, similar results being obtained with the other members of the DP family, DP-2 and DP-3. The efficiency of interaction between DP-1 and MDM2 appeared to be somewhat lower than the p53/MDM2 interaction although both interactions were specific since neither DP-1 or p53 could bind to a control GST protein. Similar experiments on the specific binding of *in vitro* translated DP-1 to a p53 fusion protein were obtained. Overall, these *in vitro* binding data support the results from the immunoprecipitation studies on the specific interaction of DP-1 with MDM2 and p53 in mammalian cells.

Mutational analysis of DP-1.

In order to determine if DP-1 and p53 can interact *in vitro* and compare the efficiency of interaction with the binding of DP-1 to E2F family members, we used an assay in which the ability of DP-1, expressed as a GST fusion protein, to bind to *in vitro* translated p53 and E2F proteins was assessed. As expected, DP-1 could associate with either E2F-1 or E2F-4 in the conditions of the assay used. Likewise, DP-1 could interact with p53, similar results being obtained with the other members of the DP family, DP-2 and DP-3. The interaction between DP-1 and p53 was as efficient as the interaction between DP-1 and E2F-1 or E2F-4, and specific since p53 failed to bind to a control GST protein. These *in vitro* binding data support the conclusion from the immunoprecipitation studies on the specific interaction of DP-1 with p53 in mammalian cells.

Using a similar assay, we determined the region in DP-1 required to bind to p53. A panel of mutant proteins derived from DP-1 representing N- and C-terminal truncations, together with a DP-1 protein altered at residues 172 and 173, were studied. *In vitro* translated wild-type DP-1 bound to p53 about 20% of the input DP-1 being retained by the wild-type p53 fusion protein. This binding efficiency was not significantly affected by removing up to 171 amino acid residues from the N-terminal region of DP-1, up to 79 amino acid residues from the C-terminal region, or by mutating residues 172 and 173. However, further N-terminal deletion from residue 171 to 205, or C-terminal deletion from residue 331 to 238, significantly reduced the binding activity of DP-1 with p53. From these data, the minimal region in DP-1 capable of efficiently binding to p53 occurs within residue 171 to 331. This region of DP-1 contains several domains which are conserved between other members of the DP family, notably DCB1 and DCB2 (Girling *et al* 1994, Ormondroyd *et al* 1995), together with the DEF box, a critical region involved in heterodimerization between DP-1 and E2F family members. Importantly, in the conditions of this assay *in vitro* translated E2F-1 failed to interact with -53 since the amount of E2F-1 retained by p53 was at background level. In the context of DRTF1/E2F, the DP protein is the principal component which is capable of interacting with p53.

An immunochemically distinct form of DP-1 associates with p53.

To define the region in p53 required for the association with DP-1, a similar *in vitro* binding assay was developed in which the ability of p53 to interact with *in vitro* translated DP-1 was monitored. The data presented above indicated that p53 coprecipitates with p55U from mammalian cell extracts and, further, that p55U is a form of DP-1 recognised by anti-DP-1 (A) but not anti-DP-1 (D). In a similar fashion, two immunochemically distinct forms of DP-1 could be defined after *in vitro* translation using these same two antisera. Specifically, in the absence of translated exogenous DP-1, anti-DP-1 (A), but not anti-DP-1- (D), recognised the endogenous DP-1 protein. After translation, both antisera recognised the *in vitro* translated DP-1 protein, the exogenous polypeptide being resolved with marginally faster mobility.

Evidence that at least two immunochemically distinct forms of DP-1 were present after *in vitro* translation was obtained upon studying the interaction with p53. When p53 was added to the *in vitro* translate, the DP-1 form recognised by anti-DP-1(A), but not anti-DP-1(D), was retained by p53 although DP-1 immunoreactive with both antisera was present in the input translate; the GST portion failed to interact with DP-1. These data indicate that two distinct forms of DP-1 are present after *in vitro* translation and further that p53 preferentially interacts with the form defined by anti-DP-1(A). Importantly, this result reflects the data derived by immunoprecipitation from mammalian cell extracts where p53 co-immunoprecipitated with p55U, a DP-1 protein recognised by anti-DP-1(A) but not anti-DP-1(D). The specificity of p53 for DP-1 in the *in vitro* binding assay therefore possesses some similarity with the interaction in mammalian cells, and supports the conclusion that p53 interacts with an immunochemcially distinct form of DP-1.

An N-terminal region in p53 is required for binding to DP-1.

We used the interaction of p53 and DP-1 to define the domain in p53 required for the association. As much as 250 amino acid residues could be deleted from the C-terminus of p53 without any detrimental effect on the interaction with DP-1. A further deletion from residue 143 to 73 abolished the interaction, thus defining a region in p53 required to bind DP-1. Since the N-terminal region of p53 contains the MDM2 binding domain (Picksley *et al*, 1994) a domain in p53 necessary for the interaction with DP-1 can therefore be distinguished from the MDM2 binding domain.

p53 and MDM2 modulate E2F site-dependent transcription.

Since DP-1 is a frequent component of DRTF1/E2F (Bandara *et al.*, 1993 and 1994), the functional consequence of an interaction of either p53 with DP-1 was assessed by studying the effects on E2F site-dependent transcription driven by DP-1 and E2F-1 a situation in which it is known that both proteins co-operate in transcriptional activation as a DNA binding heterodimer (Bandara *et al.*, 1993.

In these assay conditions DP-1 alone possesses insignificant transcriptional activity (Bandara *et al.*, 1993). When a wild-type p53 expression vector was co-transfected into 3T3 cells the level of *trans*-activation mediated by either E2F-1 alone, or DP-1 together with E2F-1, was compromised in a p53 concentration-dependent fashion. This inactivating effect of wild-type p53 was also apparent in human SAOS-2 cells which contain a mutant p53 allele. The activity of a comparable promoter construct driven by mutant E2F binding sites, p3xMT-GL, was not significantly affected by p53.

p53 and E2F-1 compete for DP-1.

It was of interest to determine if the interaction of p53 with DP-1 was mutually exclusive with the interaction of DP-1 and E2F-1. To address this question we assessed whether E2F-1, expressed as a GST fusion protein, would compete with p53 for DP-1 in the *in vitro* binding assay, conditions in which *in vitro* translated DP-1 binds to p53. As the amount of GST-E2F-1 was increased there was a concomitant reduction in the level of DP-1 bound to p53, an effect not apparent in the control GST treatment. These data indicate that p53 and E2F-1 compete for binding to DP-1, and is consistent with the earlier data indicating that p53 interacts with the dimerization domain of DP-1. If p53 and E2F-1 compete for DP-1, reduced DNA binding activity due to the DP-1/E2F-1 heterodimer may be apparent in the presence of p53. To test this possibility a band shift assay which measured the DNA binding activity of the DP-1/E2F-1 heterodimer was supplemented with p53. After *in vitro* translation DP-1 or E2F-1 alone have little DNA binding activity although, when assessed together, co-operate. As the level of p53 was titrated into the reaction, reduced DP-1/E2F-1 DNA binding activity was apparent. In contrast, inactivated p53 had little effect. In conclusion, p53 and E2F-1 compete for DP-1 and, as a result, p53 can reduce the level of DNA binding activity of the DP-1/E2F-1 heterodimer.

DISCUSSION

Distinct forms of DP-1.

It is known that DP-1 is the most widespread DNA binding component of physiological DRTF1/E2F DNA binding activity (Bandara *et al.*, 1993, 1994) where it exists in a heterodimer with an E2F family member (Bandara *et al.*, 1993; Helin *et al.*, 1993; Krek *et al.*, 1993). Previous studies have shown that the polypeptide encoded by *DP-1*, p55, is subject to cell cycle control since it is regulated during cell cycle progression, the level of p55L increasing as cells progress through the cycle (Bandara *et al.*, 1994). Using antisera which efficiently recognise p55L it has been possible to show that this form of DP-1 is the predominant DNA binding component in DRTF1/E2F, a physiological situation which is apparent in a wide variety of cell-types. In contrast, the data suggested that p55U is unlikely

to be a frequent component of DRTF1/E2F since antisera which preferentially recognise p55L react efficiently with physiological DRTF1/E2F, and the abundance of p55U inversely correlates with DRTF1/E2F DNA binding activity. Although no direct evidence that p55U and L are post-translational derivatives of each other is presented here, such an idea is a clear possibility given previous results on the effect of phosphatase on DP-1 (Bandara *et al.*, 1994).

p53 associates with DP-1.

The region in p53 required for the interaction with DP-1 exists within the N-terminal 143 amino acid residues. The first 73 residues, which contains the MDM2 binding domain are not sufficient for the interaction. Although previous studies have suggested that MDM2 can interact with DRTF1/E2F, our results imply that this interaction is unlikely to be responsible for the association of DP-1 with p53. Interestingly, the region between residue 73 and 143 which is necessary for p53 to bind DP-1 contains residues frequently altered in human tumour cells carrying mutant 53 alleles (Harris, 1993).

A variety of cellular polypeptides co-precipitated with DP-1. Two of these were identified as p53 and MDM2, proteins known to influence cell cycle progression. Thus, a property of the p53 protein is the negative regulation of the cell cycle in response to, for example, DNA damage and its gene is frequently mutated in human tumour cells (Levine *et al.*, 1991). A currently popular model for a mechanism through which p53 mediates its growth-regulating properties is that it functions as a transcriptional activator of genes involved in arresting cell cycle progression (El-Deiry *et al.*, 1993). In contrast, MDM2 is encoded by the *MDM2* oncogene, *MDM2* being amplified in certain human tumour cells (Oliner *et al.*, 1992). It is believed in part to mediate its growth promoting activity by interfering with the transcription activating properties of p53 (Oliner *et al.*, 1993).

Although it was found that p53 and MDM2 associate with DP-1, it has not been possible to detect either protein in physiological DRTF1/E2F DNA binding complexes. It is possible, given the data presented here, that these proteins target a population of DP-1 which is not frequently present in the DP-1/E2F DNA binding heterodimer, an idea which is consistent with the result that p53 co-precipitated with p55U, a form of DP-1 which is not significantly present in DRTF1/E2F DNA binding activity. Further experiments are required to clarify the significance of these observations although a possible physiological explanation may be that these interactions allow p53 and MDM2 to influence the levels of functional DP-1/E2F DNA binding heterodimers and hence transcriptionally active DRTF1/E2F, and thus regulate the downstream transcriptional activity of target genes.

Co-expression of p53 specifically inactivated transcription driven by the DP-1/E2F-1 heterodimer. Given the earlier conclusion a potential model to explain these results would be that p53 holds DP-1

in a state which prevents it interacting with an E2F family member to form a DP-1/E2F heterodimer. Indeed, the region is DP-1 required to interact with p53 is necessary to form a DP-1/E2F-1 heterodimer and thus binding of p53 to DP-1 could be mutually exclusive with the interaction of DP-1 with E2F family members. Evidence for such a possibility was obtained by demonstrating that p53 and E2F-1 can compete for DP-1 and, consequently, reduce the level of DP-1/E2F-1 DNA binding activity. These data are compatible with a model in which 53 targets an immunochemically distinct form of DP-1, regulating the formation of DP-1/E2F heterodimers and hence the level of DRTF1/E2F DNA binding activity.

The region in p53 necessary for the interaction with DP-1 incudes residues frequently altered in naturally-occurring mutant alleles. Based on the results reported here, a potential biological rationale for these mutations could be envisaged to be that they prevent p53 from interacting with DP-1 and thus relieve the negative regulation imposed by p53 on the formation of functional DRTF1/E2F and hence cell cycle progression.

A pathway for p53-mediated growth arrest.

Although p53 is believed to possess the properties of a transcription factor and the *trans* activation of target genes, such as *gadd45* and *WAF1* (Kastan *et al.*, 1992; El-Deiry *et al.*, 1993), thought to be important in p53-mediated growth arrest, the interaction of p53 with DP-1 provides another potential pathway through which p53 theoretically can influence cell cycle progression. Thus, since many of the genes regulated by DRTF1/E2F encode proteins required for cell cycle progression, their transcriptional down-regulation would be expected to impede cell cycle progression. Conversely, an increase in the activity of DRTF1/E2F may be important in mediating the physiological effects of the products of the *MDM2* oncogene.

Indeed, a variety of previous studies already have suggested that the pathways regulated by DRTF1/E2F and p53 are integrated. For example, overexpression of E2F-1 in cells induces apoptosis in a p53-dependent fashion (Wu and Levine, 1994), and increased levels of apoptosis in the lens fibre cells of $Rb^{-/-}$ mice is overcome in embryos which are doubly-null in *Rb* and *p53*. Similar conclusions have been made from studies in which the oncoproteins of tumour viruses, which can inactivate pRb or p53, are sequentially targetted to defined physiological sites (Howes *et al.*, 1994; Morgenbesser *et al.*, 1994; Pan and Griep, 1994). Overall, such studies are compatible with a model in which p53 monitors in some way the status of the DRTF1/E2F pathway. It is possible that the association between DP-1 and p53 is involved this process.

Finally, the interaction of DP-1 and p53 may help explain the mechanism through which DP-1 exerts proto-oncogenic activity, a property shared by other members of the DP family, and one manifest in the absence of a co-transfected E2F family member (Jooss *et al.*, 1995). Possibly an increased level of DP-1 sequesters p53, titrating out its activity, and thus over-riding the growth-regulating effects of p53. In this respect, DP-1 may act in an analogous fashion to certain viral oncoproteins, such as the adenovirus E1b and papilloma virus E6 proteins, since their ability to inactivate p53 correlates with oncogenic activity (Moran, 1993).

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CLAIMS

1. A complex comprising a p53 protein (a transcription modulator) and one or both of a DP protein and an E2F protein (both transcription factors).

2. A complex according to claim 1 wherein the p53 protein is wild type p53, a mutant found in tumour cells or a fragment of said wild type or mutant protein capable of forming a complex with a DP protein or an E2F protein.

3. A complex according to claim 1 or 2 wherein the DP or E2F protein is, respectively:

(a) DP-1, DP-2, DP-3 or any of E2F-1 to E2F-5;

(b) a mutant, allelic variant or species homologue of (a);

(c) a protein at least 70% homologous to (a) or (b);

(d) a fragment of any one of (a) to (c) capable of forming a complex with a MDM2 protein or a p53 protein;

(e) a fragment of any of (a) to (d) of at least 15 amino acids long; or

(f) a fusion of any of (a) to (e) with another protein.

4. A complex according to any preceding claim carrying a revealing or detectable label.

5. A complex according to any preceding claim fixed to a solid phase.

6. A composition comprising a complex according to any of claims 1 or 4 together with a carrier or a diluent.

7. A polynucleotide which comprises:

(a) a sequence encoding the proteins present in a complex as defined in any of claims 1 to 4;

(b) a sequence complementary to (a); or

(c) a sequence at least 80% homologous to a sequence in (a) or (b).

8. A polynucleotide according to claim 7 which is a DNA polynucleotide.

9. A polynucleotide according to claim 7 or 8 which, on expression, yields a complex according to any of claims 1 to 4.

10. A double stranded polynucleotide comprising a polynucleotide according to any of claims 8 to 9 and its complementary sequence.

11. A polynucleotide according to any of claims 7 to 10 carrying a revealing or detectable label.

12. A vector comprising a polynucleotide according to any of claims 7 to 10.

13. A vector according to claim 12 which is a recombinant replicable vector comprising one or more coding sequence(s) which encodes a complex as defined in any of claims 1 to 4.

14. A host cell carrying a vector according to claim 12 or 13, or carrying two vectors which separately encode a transcription modulator and transcription factor as defined in any one of claims 1 to 3.

15. A process for preparing a complex as defined in any of claims 1 to 4, the process comprising cultivating a host cell according to any of claims 14 under conditions providing for expression, of the transcription modulator and transcription factor, and recovering the expressed complex.

16. A screening assay for identifying a potential or putative chemotherapeutic agent, the assay comprising:

(A) providing the components:

(i) a DP protein and/or an E2F protein;

(ii) a transcription modulator; and

(iii) a potential or putative chemotherapeutic agent;

and bringing them into contact under conditions in which the components (i) and (ii) in the absence of (iii) form a complex: and

(B) measuring the extent to which component (iii) is able to disrupt or interfere with the complex, inhibit or encourage the binding of components (i) and (ii), or the effect on the activity of the complex.

17. An assay according to claim 16 wherein both a DP protein and E2F protein are present and the complex of (i) and (ii) is measured by its ability to bind an E2F DNA binding site *in vitro*.

18. An assay according to claim 16 wherein the component (i) is a complex as defined in any of claims 1 to 5 and the interaction or binding of components (i) and (ii) is measured by the ability of the complex to activate *in vivo* a promoter comprising an E2F binding site linked to a reporter gene.

19. An assay according to claim 18 wherein the assay is performed in a yeast cell, insect cell or a mammalian cell.

20. An assay according to any of claims 16 to 19 wherein the chemotherapeutic agent is a fragment of 10 or more amino acids of a protein as defined in parts (a) to (e) of claim 3.

21. A screening assay for identifying a potential or putative chemotherapeutic agent, the assay comprising providing a transcription modulator or a transcription factor, and a potential or putative chemotherapeutic agent, and measuring the extent to which the agent binds to the modulator or factor, or whether the modulator or factor form a complex with the agent.

22. A complex as defined in any of claims 1 to 4, a polynucleotide as defined in any of claims 7 to 11, a vector as defined in claim 12 or 13 or a host cell as defined in claim 14 for use in a method of treatment of the human or animal body.

23. A pharmaceutical composition comprising a complex as defined in any of claims 1 to 4, a polynucleotide as defined in any of claims 7 to 11, a vector as defined in claim 12 or 13 or a host cell as defined in claim 14 for use in a method of treatment of the human or animal body and a pharmaceutically acceptable carrier or excipient.

24. The use of:

(a) a complex as defined in any of claims 1 to 4;

(b) a polynucleotide as defined in any of claims 7 to 11;

(c) a vector as defined in claims 12 or 13; or

(d) a host cell as defined in claim 14;

in the manufacture of a medicament for treating a proliferative disease.

INTERNATIONAL SEARCH REPORT

[redacted] al Application No
[redacted]T/GB 96/01560

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/83 C12N15/12 C12N5/10 C07K19/00 C07K14/82
C07K14/47 G01N33/68 A61K38/17 //G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROC. NATL ACAD. SCI., vol. 91, no. 9, 26 April 1994, pages 3602-3606, XP002012349 WU ET AL.: "p53 and E2F-1 cooperate to mediate apoptosis" see the whole document	7,8,11, 12,14
Y		1-4,9, 10,15-24

X	PROC. NATL ACAD. SCI., vol. 91, no. 23, 8 November 1994, pages 10918-10922, XP002012350 QIN ET AL.: "Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis" see the whole document	7,8,12, 14
Y		1-4,9, 10,15-24

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
2 September 1996	13. 09. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gac, G

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INTERNATIONAL SEARCH REPORT

Internat[illegible] l Application No
P[illegible]B 96/01560

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, vol. 375, no. 6533, 22 June 1995, pages 691-694, XP002012351 MARTIN ET AL.: "Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein" see the whole document	16-18,21
X	WO,A,94 00603 (THE TRUSTEES OF PRICETON UNIVERSITY) 6 January 1994 see page 19 - page 21	21
Y	J. VIROLOGY, vol. 68, no. 5, May 1994, pages 2811-2821, XP002012352 VON KNEBEL DOEBERITZ ET AL.: "Reversible repression of Papillomavirus oncogene expression in cervical carcinoma cells : consequences for the phenotype and E6-p53 and E7-pRB interactions" see abstract see page 2816 - page 2817 see page 2818; figure 9 see page 2819	16
P,X	EMBO J., vol. 14, no. 24, 15 December 1995, pages 6184-6192, XP002012353 O'CONNOR ET AL.: "Physical and functional interactions between p53 and cell cycle co-operating transcriptional factors, E2F1 and DP1" see the whole document	1-24
A	CRIT. REV. EUKARYOT. GENE EXPR., vol. 5, no. 3-4, 1995, pages 365-383, XP000601475 MEYERS ET AL.: "Indirect and direct disruption of trancriptional regulation in cancer : E2F and AML-1" see page 365 - page 370	1-24
A	SEMIN. CANCER BIOLOGY, vol. 6, April 1995, pages 99-108, XP000601456 ADAMS ET AL.: "Transcriptional control by E2F" see page 104 see page 106	1-24

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/01560

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. CELL. SCI., vol. 19, 1995, pages 91-94, XP000601457 MARTIN ET AL.: "Regulation of transcription by E2F1/PD1" see the whole document ---	1-3,23, 24
A	PROC. NATL ACAD. SCI., vol. 92, no. 12, 6 June 1995, pages 5436-5440, XP002012354 ALMASAN ET AL.: "Deficiency of retinoblastoma protein leads to inappropriate S-phase entry, activation of E2F-responsive genes, and apoptosis" see the whole document -----	1-24

INTERNATIONAL SEARCH REPORT

[redacted] patent family members

Internat[redacted] Application No
PCT[redacted] 96/01560

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		CA-A-	2137822	06-01-94
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		JP-T-	8500729	30-01-96
		WO-A-	9400601	06-01-94